# EARLY EXPRESSION OF ANGIOGENESIS FACTORS IN ACUTE MYOCARDIAL ISCHEMIA AND INFARCTION

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## ABSTRACT

***Background*** When the myocardium is deprived of blood, a process of ischemia, infarction, and myocardial remodeling is initiated. Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator of vascular endothelial growth factor (VEGF) and is critical for initiating early cellular responses to hypoxia. We investigated the temporal and spatial patterns of expression of the $\alpha$ subunit of HIF-1 (HIF-1$\alpha$) and VEGF in specimens of human heart tissue to elucidate the early molecular responses to myocardial hypoxia.

***Methods*** Ventricular-biopsy specimens from 37 patients undergoing coronary bypass surgery were collected. The specimens were examined by microscopy for evidence of ischemia, evolving infarction, or a normal histologic appearance. The specimens were also analyzed with the reverse-transcriptase polymerase chain reaction for HIF-1$\alpha$ and VEGF messenger RNA (mRNA) expression and by immunohistochemical analysis for the location of the HIF-1$\alpha$ and VEGF proteins.

***Results*** HIF-1$\alpha$ mRNA was detected in myocardial specimens with pathological evidence of acute ischemia (onset, <48 hours before surgery) or early infarction (onset, <24 hours before surgery). In contrast, VEGF transcripts were seen in specimens with evidence of acute ischemia or evolving infarction (onset, 24 to 120 hours before surgery). Patients with normal ventricles or evidence of infarction in the distant past had no detectable levels of either VEGF mRNA or HIF-1$\alpha$ mRNA. HIF-1$\alpha$ immunoreactivity was detected in the nuclei of myocytes and endothelial cells, whereas VEGF immunoreactivity was found in the cytoplasm of endothelial cells lining capillaries and arterioles.

***Conclusions*** An increase in the level of HIF-1$\alpha$ is an early response to myocardial ischemia or infarction. This response defines, at a molecular level, one of the first adaptations of human myocardium to a deprivation of blood. HIF-1$\alpha$ is a useful temporal marker of acutely jeopardized myocardium. (N Engl J Med 2000;342:626-33.)



HYPOXIA is a potent regulator of a variety of biologic processes, including angiogenesis, vascular contractility, and erythropoiesis.[1-4] When a coronary artery is partially or totally occluded, metabolic and contractile changes are initiated in the heart within seconds.[5] Some of these early changes facilitate cellular preservation and functional survival of the heart. If the myocardium remains deprived of blood, changes of progressively greater severity eventually culminate in cell death, tissue necrosis, and myofibrillar remodeling.[6]

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional factor that is expressed in response to a decrease in the partial pressure of cellular oxygen and activates genes involved in angiogenesis, glycolysis, modulation of vascular tone, and erythropoiesis.[7-11] It is a heterodimer composed of $\alpha$ and $\beta$ subunits, both of which are members of the family of basic helix–loop–helix peptides.[12] HIF-1$\alpha$ is an 826-amino-acid protein that functions as a *trans*-acting transcriptional activator of vascular endothelial growth factor (VEGF), inducible nitric oxide synthase, lactate dehydrogenase, and erythropoietin.[13-16] HIF-1$\beta$ is a constitutively expressed nuclear translocator protein that forms heterodimers with HIF-1$\alpha$ as well as other nuclear proteins.[17]

Several studies have found increased levels of HIF-1$\alpha$ messenger RNA (mRNA) in hypoxic cultured cells and in organs (the retina and lung) of animals exposed to short- or long-term hypoxia.[18-20] We hypothesized that an increase in the steady-state levels of HIF-1$\alpha$ mRNA is one of the earliest responses to myocardial ischemia and infarction in humans and that it potentially is an important stimulus of angiogenesis and myocardial-cell survival. To investigate this possibility, we examined HIF-1$\alpha$ mRNA expression in relation to changes in steady-state levels of VEGF mRNA. VEGF is an inducible factor that controls capillary growth and angiogenesis in several organ systems.[21] The temporal and spatial patterns of expression of HIF-1$\alpha$ and VEGF proteins were also studied to identify molecular markers of the myocardial response to ischemia.

## METHODS

### Selection of Patients

Between November 1997 and April 1998, 37 patients (27 men and 10 women; mean age, 65.9 years; range, 55 to 75 years) who were undergoing coronary bypass surgery were enrolled in the study. Seven of these patients had a clinical history, electrocardiographic findings, and creatine kinase and troponin I levels indi-

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cating that myocardial infarction had occurred within the preceding 24 hours (early infarction); 8 patients had evidence, on the basis of the same variables, that myocardial infarction had occurred during the preceding 24 to 120 hours (evolving infarction); and 10 patients had evidence of myocardial ischemia of less than 48 hours' duration (acute ischemia), defined as angina or heart failure without Q waves on the electrocardiogram and without an increase in serum levels of creatine kinase and troponin I. Twelve patients underwent coronary bypass surgery but had not had angina or heart failure within the preceding 10 days. According to usual practice at our institution, myocardial infarction was defined as a total creatine kinase level of more than 150 U per liter (normal range, 10 to 150) or a troponin I level of more than 0.6 ng per milliliter (normal range, less than 0.6).[22]

The criteria for enrollment included the need for urgent or elective coronary bypass surgery, an age between 55 and 75 years, and written informed consent for heart biopsy. The study was approved by the University of California, San Diego, institutional review board.

### Biopsy of Myocardium

After the induction of anesthesia and median sternotomy, the heart of each patient was examined, and 3-mm, partial-thickness biopsy specimens were taken from the left ventricle. In patients with early or evolving infarction, specimens were taken from the area of presumed infarction as well as from an area of the ventricle free of coronary disease that could have caused ischemia or infarction. Likewise, in patients with acute ischemia, specimens were taken from the area of ischemia as well as from an area of normal ventricular tissue. In this way, each patient with ischemia or infarction served as his or her own control. In patients without evidence of ischemia or infarction, a single ventricular-biopsy specimen was obtained. All biopsies were performed before cardiopulmonary bypass, during ventilation with a fraction of inspired oxygen of 40 percent and peripheral oxygen saturations of greater than 95 percent. Biopsy sites were closed with polypropylene sutures.

### Extraction of RNA and Analysis of Specimens

Half of each biopsy specimen was fixed in formalin, sectioned to a thickness of 5 $\mu$m, mounted on slides, and stained with hematoxylin and eosin. The mounted specimens were then examined for evidence of acute ischemia and early or evolving infarction.[23] The other half of each specimen was frozen in liquid nitrogen at −140°C. Portions of the frozen samples were lyophilized, and then RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform technique, as previously described.[24,25]

The recovered RNA pellet was dried under vacuum conditions for 10 to 15 minutes and then dissolved in diethyl pyrocarbonate–treated deionized distilled water. The concentration and purity of the RNA were determined by spectrophotometric analysis (Ultrospec II, Biochrom, Cambridge, England) at 260 and 280 nm. The samples were stored at −80°C until analyzed.

### Measurement of RNA

The reverse-transcriptase polymerase chain reaction (PCR) was used to analyze each ventricular specimen for the presence of transcripts encoding HIF-1$\alpha$, HIF-1$\beta$, VEGF, and cyclophilin. Cyclophilin mRNA was studied as a marker to control for variation in RNA concentration and RNA degradation as potential confounding variables. Five micrograms of total RNA was used to synthesize complementary DNA (cDNA) with Super Script II Reverse Transcriptase and oligo(dT)$_{12\text{-}18}$ (GIBCO BRL, Gaithersburg, Md.). The synthesized primers had the following sequences: for HIF-1$\alpha$, 5′CTGTGATGAGGCTTACCATCAGC3′ and 5′CTCGGCTAGTTAGGGTACACTTC3′; for HIF-1$\beta$, 5′CAGGTCGGATGATGAGCAGAGCA3′ and 5′CTCATGGAAGACTGCTGACCTTC3′; for VEGF, 5′GGATGTCTATCAGCGCAGCTAC3′ and 5′TCACCGCCTCGGCTTGTCACATC3′; and for cyclophilin, 5′GTGACTTCACACGCCATAATGGC3′ and 5′GGTGCTCTCCTGAGCTACAGAAGG3′.

Duplicate amplification reactions were carried out with a single-block thermocycler (Ericomp, San Diego, Calif.) containing 2 $\mu$l of first-strand cDNA, 1 $\mu$l of each primer, deoxynucleotide triphosphates (at 10 mM each), 25 mM magnesium chloride, 0.5 U of *Taq* DNA polymerase, and 36.5 $\mu$l of autoclaved distilled water. Each sample underwent initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR products were electrophoresed on 1.8 percent agarose gels containing 3 percent ethidium bromide in TRIS–acetate–EDTA buffer. The gels were photographed with an electrophoresis photodocumentation camera (Fisher Scientific, Pittsburgh) on black-and-white film (3000ISO, Polaroid, Cambridge, Mass.).

### Immunohistochemical Staining for HIF-1$\alpha$ and VEGF

Portions of the frozen biopsy specimens were fixed in 10 percent formalin and prepared as 5-$\mu$m-thick tissue sections on slides. The paraffin was then removed with a xylene substitute (Hemo-De, Fisher Scientific) and the sections were rehydrated with ethanol gradient washes. The sections of affected tissue and normal tissue from patients with ischemia or infarction and the sections from patients without ischemia or infarction were incubated with either mouse antihuman HIF-1$\alpha$ IgG (IgG2v, Novus Biological, Littleton, Colo.) or mouse antihuman VEGF IgG ($IgG_{2a}$, Santa Cruz Biotechnology, Santa Cruz, Calif.) at a 1:100 dilution. Control sections were incubated with diluted normal horse serum (Vector Laboratories, Burlingame, Calif.) instead of the primary antibody. All the sections were subsequently incubated with biotinylated secondary antimouse antibodies and stained with an immunoperoxidase technique (Vectastain Elite ABC reagents, Vector Laboratories). Sections were dried and mounted (Gel Mount, Biomeda, Foster City, Calif.), examined with a photomicroscope, and photographed on color film (Fujicolor 100, Tokyo, Japan).

### Statistical Analysis

Data for each continuous variable were examined with the Shapiro–Wilk W test to determine whether assumptions of normality were valid. When continuous variables were compared among the groups of patients, an independent Student's t-test and one-way analysis of variance were used for normally distributed data, and the Wilcoxon rank-sum test and the Kruskal–Wallis test were used for non-normally distributed data. For all the tests, the significance level was 5 percent. When significant differences were found among the groups, pairwise comparisons were made to identify the source of the differences. Overall type I error rates of 5 percent were controlled with use of the Tukey–Kramer honestly-significant-differences test (with analysis of variance) or the Nemenyi test (with the Kruskal–Wallis test). Descriptive data for continuous variables are reported as means ±SD or as medians and ranges. Categorical variables are presented as numbers and percentages. Data were analyzed with JMP software (version 3.2.1, SAS Institute, Cary, N.C.) or with a program written in S+ (version 5.0, release 3, MathSoft, Seattle).

## RESULTS

### Classification of Ventricular Specimens and Preoperative Characteristics of the Patients

All ventricular-biopsy specimens were examined by light microscopy for evidence of ischemia or infarction by a cardiac pathologist who was unaware of the patients' identity. Seven specimens had pathological evidence of acute myocardial infarction that had occurred less than 24 hours before biopsy (designated the early-infarction group), 8 specimens had evidence of acute infarction that had occurred 24 to 120 hours before biopsy (the evolving-infarction group),

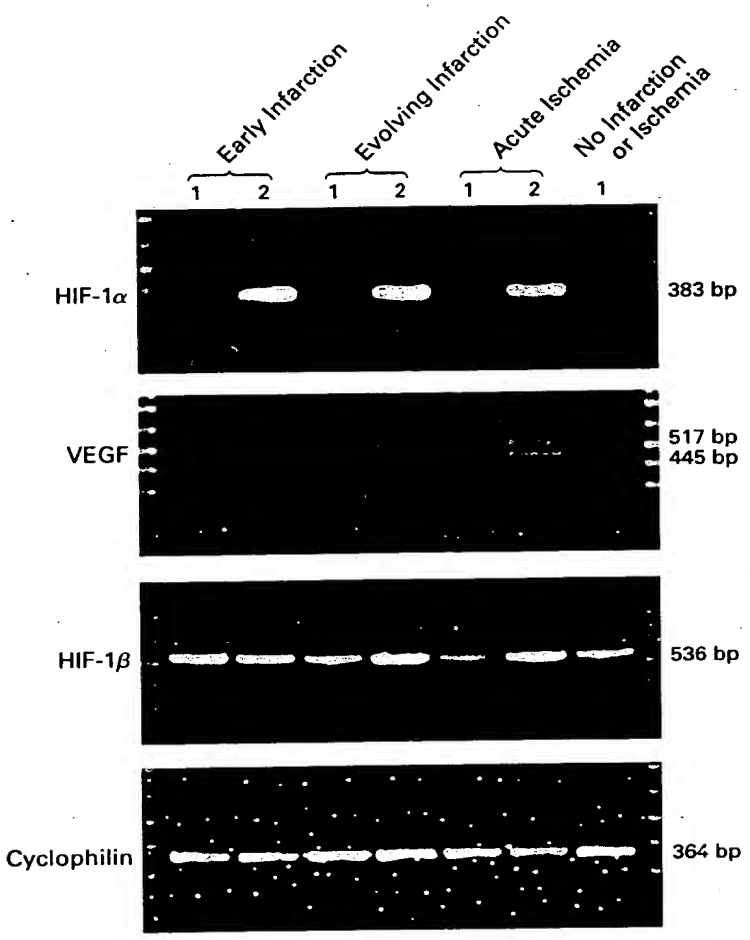


**Figure 2.** Results of Analysis of Ventricular-Biopsy Specimens by the Polymerase Chain Reaction.

In specimens in the early-infarction group, the onset of infarction was less than 24 hours before surgery; in those in the evolving-infarction group, the onset of infarction was 24 to 120 hours before surgery; and in those in the ischemia group, the onset of ischemia was less than 48 hours before surgery. In each pair, 1 denotes the specimens taken from an area of normal ventricular tissue, and 2 the specimens taken from an area of ischemic or infarcted ventricular tissue. The lengths of the transcripts detected are shown on the right.

protein in these specimens was found only in the cytoplasm of endothelial cells lining the small vessels and was not present in cardiomyocytes (Fig. 3). VEGF protein was not detected in specimens with early infarction but was detected in specimens with evolving infarction or specimens with ischemia, in which it was confined to the myocardial vasculature. We did not detect HIF-1$\alpha$ protein or VEGF protein by Western blot analysis of the peripheral blood of any of the patients (data not shown), suggesting that these proteins and their effects are confined to the heart.

## DISCUSSION

To survive periods of stress and ischemia, the human heart has developed mechanisms to adapt to changes in its environment. One of these mechanism is the ability to promote growth of new blood ves sels into ischemic areas, thus limiting regions of im pairment and ultimately preserving myocardial func tion.[26] The decrease in the partial pressure of cellula oxygen induced by ischemia is a potent stimulator c neovascularization in several organ systems. Semer za has shown in both in vitro and in vivo models c ischemia that one of the first genes up-regulated b hypoxia is the gene encoding HIF-1.[7] HIF-1 protei is composed of two distinct peptides. Expression c the gene for HIF-1$\alpha$ is exquisitely sensitive to the or set of cellular hypoxic conditions, making it one of th earliest effectors of the response to ischemia.[27] HI 1$\beta$, the other component of the HIF-1 protein, is

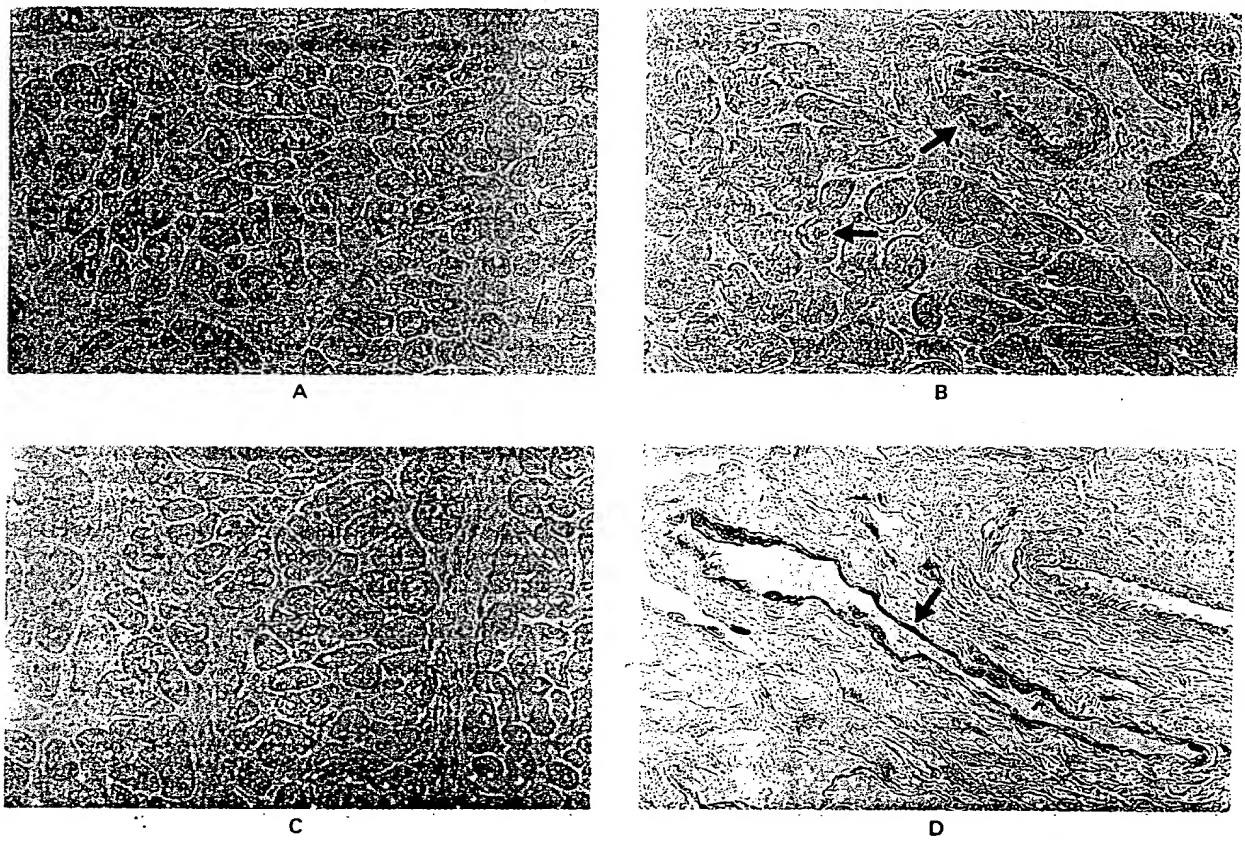


**Figure 3.** Localization of HIF-1$\alpha$ and VEGF Proteins in Ventricular-Biopsy Specimens (Immunchistochemical Staining, ×400).
Immunohistochemical analysis of ischemic or infarcted ventricular-biopsy specimens was performed on permanent sections without antibody to HIF-1$\alpha$ (Panel A), with antibody to HIF-1$\alpha$ (Panel B), without antibody to VEGF (Panel C), and with antibody to VEGF (Panel D). The results show localization of HIF-1$\alpha$ in the nuclei of cardiomyocytes and endothelial cells in the section analyzed with antibody to HIF-1$\alpha$ (Panel B, arrows) and localization of VEGF in the cytoplasm of endothelial cells in the section analyzed with anti-VEGF antibody (Panel D, arrow).

high-affinity protein that binds to HIF-1$\alpha$ in the cytosol and transports HIF-1$\alpha$ into the nucleus, where HIF-1$\alpha$ may exert its *trans*-acting effect.[28] Expression of HIF-1$\beta$ is constitutive, not sensitive to hypoxia, in several types of tissue culture and in solid organs.[29]

After it is activated by a low partial pressure of cellular oxygen, HIF-1 binds to a specific hypoxia-responsive element in the regulatory regions of several hypoxia-sensitive genes, leading to their transcriptional activation. We hypothesized that one of the most crucial actions of HIF-1 is to regulate the gene encoding the angiogenesis factor VEGF and thus ultimately to trigger the cascade of angiogenesis.

The goal of this study was to examine specimens of human heart tissue affected by various degrees of ischemic insult and to correlate the physiologic and pathological state of the heart with the temporal and spatial expression of HIF-1 and VEGF. In our patients, we detected increased steady-state levels of HIF-1$\alpha$ mRNA during the early period (the first 24 hours) after acute myocardial infarction or during acute myocardial ischemia. This accumulation of mRNA was limited to the region of affected myocardium. No HIF-1$\alpha$ transcripts were detectable by PCR analysis in specimens of nonischemic or noninfarcted tissue. These results suggest that HIF-1$\alpha$ is an early molecular marker of myocardial ischemia or infarction. The production of this protein and its effects appear to be limited to the heart, since it was not detected in the peripheral blood of our patients.

Immunoreactivity to HIF-1$\alpha$ was detected in both myocardial and endothelial cells in all specimens of human heart affected by ischemia or infarction. Since the half-life of HIF-1$\alpha$ protein has been estimated to be on the order of minutes,[30] we surmise that HIF transcription or stabilization of HIF mRNA contin-

ues throughout early ischemia or infarction to generate protein at levels that are detectable by antibody staining.

Two important limitations of this study should be recognized. First, by using the PCR to detect steady-state levels of HIF-1$\alpha$ and VEGF mRNA, we did not measure the amount of transcript present in each tissue specimen. Rather, we determined whether mRNA was present or absent at the defined sensitivity of the assay (1 or more mRNA molecules per 1000 cells).[31] Our study did not distinguish whether the spatial and temporal accumulation of HIF-1$\alpha$ and VEGF mRNA in the heart reflected enhanced transcription or enhanced stabilization of mRNA. Evidence from cultured cell lines[32,33] and animal models[34] suggests that both mechanisms may be important in the regulation of hypoxia-sensitive genes. Second, since our study examined specimens from human subjects, it was necessarily limited in scope and time course. Despite our inability to perform serial biopsies in individual patients, we found clear evidence that HIF-1$\alpha$ expression is confined to the region of acute hypoxia in the heart; that it is initiated within hours of the onset of myocardial ischemia or infarction, or even earlier; and that detectable levels of HIF-1$\alpha$ mRNA are transient. Our results suggest that HIF-1 induced by ischemia may be a signal mechanism for controlling the early-to-intermediate expression of genes that initiate angiogenesis during myocardial hypoxia.

VEGF has an important role in stimulating the growth of new capillaries in several organ systems and thus is a good candidate for the role of stimulating neovascularization to limit damage from infarction in the heart. Although the mechanism of enhanced VEGF expression remains to be determined, it is worth noting that the gene for VEGF has an HIF-1 regulatory consensus sequence (a hypoxia-responsive element) in its promoter region.[35] These observations, together with the previous finding that HIF-1 is responsible for the increase in VEGF in cultured hypoxic myocytes,[36] suggest that the increase in myocardial HIF-1 protein that we detected in ischemic and infarcted tissue is necessary, at least in part, for the enhanced expression of myocardial VEGF in states of ischemia.

We found in specimens of human heart tissue that steady-state levels of VEGF mRNA were present in the initial periods of ischemia (<48 hours after onset) and in the intermediate periods of infarction (24 to 120 hours after onset). Expression of VEGF persisted for a longer time after the onset of myocardial ischemia or infarction than did HIF-1$\alpha$ expression. This suggests that the response of HIF-1$\alpha$ to ischemia occurs early and is transient, whereas the VEGF response is of longer duration and is probably necessary for preservation of the myocardium and limitation of hypoxic cellular destruction. VEGF protein in the myocardium was found only in the endothelium that lined medium and small arterioles and capillaries, in contrast to HIF-1 protein, which was expressed in both vascular endothelial cells and myocardial cells. This observation suggests that the angiogenic effects of HIF-1 and VEGF are limited to regions of terminal small vessels in the myocardium. The importance of the difference between the types of cells that express HIF-1$\alpha$ (endothelial and myocardial cells) and those that express VEGF (endothelial cells) remains to be determined. It is possible that in myocardial cells HIF-1$\alpha$ controls hypoxia-responsive genes other than the gene encoding VEGF.

In conclusion, we defined at a molecular level the sequential expression of HIF-1$\alpha$ and VEGF in the human heart during ischemia. The genes encoding these two proteins are molecular temporal and spatial markers of ischemic myocardium. The presence of HIF-1$\alpha$ mRNA and subsequently the presence of VEGF mRNA in the heart tissue of patients with infarction provide compelling new evidence that HIF-1$\alpha$ contributes to limitation of infarct size by promoting angiogenesis and vascular remodeling and that it does so by increasing steady-state levels of VEGF mRNA. The expression of HIF-1$\alpha$ by both myocardial cells and endothelial cells in the hypoxic heart raises the possibility that HIF-1$\alpha$ has a broad role in myocardial disease associated with ischemia and infarction. Although elucidation of the pathophysiologic importance of HIF-1$\alpha$ in these conditions awaits the availability of specific HIF-1 antagonists, the present study provides new information about variations in local synthesis and distribution of HIF-1 and VEGF in human heart disease. Further elucidation of the effects of these proteins may reveal clues for approaches to limiting infarct size and the sequelae of hypoxic damage to the myocardium.

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